REMARKS

Claims 287-305 were previously pending in this application. In the complete listing of claims above, claims 290-292, 297 and 300 have been amended and new claims 306-307 have been added. No other claims have been amended, added or canceled by this paper. Accordingly, as reflected in the above claim listing, claims 287-307 are presented for further examination.

Amendments to the Claims

As just indicated, claims 290-292, 297 and 300 have been amended. Claim 290 depends from claim 289 and ultimately from claim 287. Claim 290 now recites that "said substituted aliphatic group comprises halogen *or* sulfonates.[1] Thus, only two members are now claimed in claim 290. Claim 291 depends from claim 287 and recites "wherein said enzymatic converting step (c) is carried out by a substrate comprising glucose, xylose, fucose, amino acids, *or esters of phosphates, carboxylic acids or fatty acids*.[2] Thus, the term "amides" has been deleted from claim 291 and the "phosphates, carboxylic acids and fatty acids" have been limited to their *esters*. In claim 292, several of the listed enzymatic activities have been deleted. Thus, as amended above, claim 292 recites "wherein said enzymatic activity of interest comprises an amidase, a trypsin or a chymotrypsin."

The structure recited in step (a) (ii) in claim 297 has been amended to depict the groups R and R' as separate ring substituents. This change was precipitated by

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[1] Originally, claim 290 recited "wherein said substituted aliphatic group comprises halogen, nitrates, sulfonates or nitrates."

[2] Originally, claim 291 recited "wherein said enzymatic converting step (c) is carried out by a substrate comprising amides, esters, phosphates, carboxylic acids, fatty acids, glucose, xylose, fucose, or amino acids."

the indefiniteness rejection under 35 U.S.C. §112, second paragraph (see September 8, 2006 Office Action, page 2, no. 1).

Finally, claim 300 has been amended in largely the same manner as claim 290. Claim 300 now recites "wherein said substituted aliphatic group comprises halogen or sulfonate."[3] Again and as in the case of claim 290, two previously recited members in claim 300 have been deleted.

It is believed that the foregoing amendments do not insert new matter into Applicants' disclosure.

Entry of the above amendments is respectfully requested.

New Claims

As set forth on page 4 in the September 8, 2006 Office Action, claims 295-296 were deemed allowable if rewritten in independent form including all the limitations in the base claim and intervening claims. Thus, new claims 306 and 307 have been added above, and these correspond to claims 295 and 296, respectively, except that base claim and intervening claim limitations have been included. New claim 306 recites in its preamble "[a] process for detecting the presence or quantity of enzymatic activity of interest in a sample, *said enzymatic activity being dependent upon the presence or quantity of another compound*, . . . In the first step (a) of claim 306, a sample (i) is provided that is suspected of containing enzymatic activity "*that is dependent upon the presence or quantity of said another compound.*" New claim 307 recites in its preamble "[a] process for detecting the presence or quantity of enzymatic activity of interest in a sample, *said enzymatic activity being dependent upon the presence or quantity of an RNA or DNA probe*, . . ." In the first step of claim 307, a sample is provided that is suspected of containing enzymatic activity *that is dependent upon the presence or*

[3] Originally, claim 300 recited "wherein said substituted aliphatic group comprises halogen or esters of nitrate, sulfonate or nitrite."

quantity of an RNA or DNA probe." Because claims 306 and 307 are merely claims rewritten with the limitations of the base claim and the intervening claims, no new matter is inserted by their presentation.

Entry of new claims 306 and 307 is respectfully requested.

The First Rejection Under 35 USC §112, Second Paragraph

Claims 297-305 stand rejected for indefiniteness under 35 U.S.C. §112, second paragraph. In the Office Action (page 2), it is stated:

> Claim 295 shows a structure of a chemiluminescent reagent comprising an aromatic moiety having R and R' substituents. However it is unclear if the R substituent is attached to the ring or if R and R' are linked and form only one substituent attached to the ring.

The indefiniteness rejection is respectfully traversed.

As indicated above, claim 297 has been amended above to show the groups R and R' as separate ring substituents.

In view of the foregoing amendment to claim 297, Applicants respectfully request reconsideration and withdrawal of the indefiniteness rejection of record.

The Rejection Under 35 USC §102(b)

Claims 287-292 and 294 stand rejected under 35 U.S.C. §102(b) as being anticipated by Bronstein et al., U.S. Patent No. 5,800,999. The text of the anticipation rejection provided below is found on pages 2-4 in the September 8, 2006 Office Action.

> Bronstein et al. discloses a 1, 2-dioxetane compound where, T is a stabilizing group (adamantly). The adamantyl group, spiro-bound, can be substituted at any bridge head carbon, to affect chemiluminescent properties. The remaining carbon of the dioxetane ring bears a OR substituent, wherein R is generally an alkyl or cycloalkyl, although it may be a further aryl group. Preferred embodiments include substituted alkyls, with the substituent including

halogenated groups, such as polyhaloalkyl substituents. The remaining valence is occupied by an aryl moiety, preferably phenyl or naphthyl. If naphthyl, particular substitution profiles on the naphthyl ring are preferred. The aryl ring bears at least one substituent, X. In commercially developed dioxetanes, this is an enzyme-cleavable group. For instance, many assays employ an exogenous enzyme, such as alkaline phosphatase, to ensure reliability of the assay. The enzyme is typically conjugated to a binding ligand, either an antibody, a nucleic acid fragment, or similar binding pair member, which will bind to the target substance to be detected. Where the conjugated enzyme is alkaline phosphatase, the enzyme-cleavable group X will be a phosphate. The aryl ring may also bear a substituent Y, which is selected to be either electron donating, or electron withdrawing. Preferred groups include chlorine, alkoxy and heteroaryl, although other groups may be employed. These substitutions further effect chemiluminescent properties, and reaction kinetics.

Uniformly, these dioxetanes are disclosed as useful enzyme substrates, that is, the binding pair member conjugated to an enzyme is allowed to bind to the target analyte, and after washing to remove unbound material, the dioxetane is added. In the presence of the conjugated enzyme, the protective group is cleaved, leading to decomposition of the dioxetane, and light emission. The thermal stability of the dioxetanes is superior to that of radioisotopes, fluorophores and other available chemiluminescent systems. Because biological assay conditions generally employ an aqueous media, water solubility, an important criteria, was met by use of the dioxetane substrates, which proved easy to use in both qualitative and quantitative determinations, in solutions, and in blotting assays. (col.2)

The anticipation rejection is respectfully traversed.

It is believed that Bronstein's patent does not anticipate the present invention because it lacks a material element. More particularly, the present invention calls for the enzymatic conversion of R_1 into R_1* which comprises a chemical reactive group G_1. An unstable light-emitting form of dioxetane is only formed in the present invention when a chemically reactive group G_2 (which is part of R_2) participates in an intramolecular chemical reaction with G_1 which has been formed by the enzymatic conversion of R_1 into R_1*. In contrast, there is no

mention or suggestion in Bronstein et al. that after the enzymatic reaction, an intramolecular conversion could or should take place before forming the unstable dioxetane.

Allowable Subject Matter

Applicants appreciate the indication in the Office Action (page 4) that claims 295-296 were objected to as being dependent upon a rejected base claim, but would be allowable if rewritten in independent form including all of the limitations of the base claim and any intervening claims.

In response and as indicated earlier, new claims 306-307 have been added with the limitations of the base claims and intervening claims having been included.

An early indication of the allowability of claims 306-307 is respectfully requested.

Previously Submitted References

In the Office Action (page 4), the Examiner indicated that the references in the PTO-1449 were lined through because they were not provided or were missing a publication date.

In response, Applicants are providing as Exhibit A to this paper a copy of Dale et al., "Direct Covalent Mercuration of Nucleotides and Polynucleotides," Biochemistry 14:2447-2457 (1975). Dale et al. was provided as Exhibit 10 to Applicants' March 8, 2004 Information Disclosure Statement.

It is respectfully requested that Dale et al. be made of record in this application and considered in determining the patentability of any and all claims.

Early and favorable action is respectfully requested.

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Enz-61(D11)

SUMMARY AND CONCLUSIONS

In the claim listing above, claims 287-307 are presented for further examination. Of these, claims 290, 291, 292, 297 and 300 have been amended and new claims 306-307 have been added.

The fee for adding new claims 306-307 is $200 based upon the presentation of one new independent claims above the three previously paid for independent claims. No other fee or fees are believed due in connection with this paper. In the event that any fee or fees are due, however, the Patent and Trademark Office is hereby authorized to charge any such fee or fees to Deposit Account No. 05-1135, or to credit any overpayment thereto.

If a telephone conversation would further the prosecution of the present application, Applicants' undersigned attorney requests that he be contacted at the number provided below.

Respectfully submitted,

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Stavrianopoulos et al., Serial No. 10/764,389 (Filed January 23, 2004)
Exhibit A [To Applicants' October 19, 2006 Amendment Under 37 C.F.R. §1.115]

EXHIBIT A

[Reprinted from Biochemistry, (1975) 14, 2447.]

Direct Covalent Mercuration of Nucleotides and Polynucleotides†

R. M. K. Dale,* E. Martin,‡ D. C. Livingston,§ and D. C. Ward

ABSTRACT: Nucleotides of cytosine and uracil are readily mercurated by heating at 37–50° in buffered aqueous solutions (pH 5.0–8.0) containing mercuric acetate. Proton magnetic resonance, elemental, electrophoretic, and chromatographic analyses have shown the products to be 5-mercuricytosine and 5-mercuriuracil derivatives, where the mercury atom is covalently bonded. Polynucleotides can be mercurated under similar conditions. Cytosine and uracil bases are modified in RNA while only cytosine residues in DNA are substituted. There is little, if any, reaction with adenine, thymine, or guanine bases. The rate of polymer mercuration is, unlike that of mononucleotides, markedly influenced by the ionic strength of the reaction mixture: the lower the ionic strength the faster the reaction rate. Pyrimidine residues in single- and double-stranded polymers react at essentially the same rate. Although most polynucleotides can be extensively mercurated at pH 7.0 in sodium or Tris-acetate buffers, tRNA undergoes only limited substitution in Tris buffers. The mild reaction conditions give minimal single-strand breakage and, unlike direct iodination procedures, do not produce pyrimidine hydrates. Mercurated polynucleotides can be exploited in a variety of ways, particularly by crystallographic and electron microscopic techniques, as tools for studying polynucleotide structure.

The formation of coordination complexes between mercuric ions and nucleotides or polynucleotides has been known for over 20 years (Katz, 1952, 1963; Thomas, 1954; Yamane and Davidson, 1961; Simpson, 1964; Nandi et al., 1965; Gruenwedel and Davidson, 1967; Mansy et al., 1974). These complexes are quasistable, readily reversed by the addition of agents that act as ligands for Hg^{2+}, such as Cl^- and CN^-, and involve both valences of the Hg^{2+} ion. Although the structure of the complexes were never fully elucidated, Hg^{2+} binding appeared to result from interaction with amino groups and ring nitrogens of the bases (Katz, 1963; Yamane and Davidson, 1961), preferential binding occurring with A-T base pairs (Nandi et al., 1965). Davidson and associates (Nandi et al., 1965; Wang et al., 1965) utilized this selective binding to induce large buoyant density differences in Cs_2SO_4 between DNAs with different base compositions, or between single- and double-stranded DNA. Covalent[1] mercuripolynucleotides could have even greater utility in the selective separation of polymers, in the structural analysis of polynucleotides or polynucleotide–protein complexes, or in electron microscopic methods of gene mapping, provided the mercury substituents are (1) reasonably stable and (2) do not significantly distort the polymer structure. The high electron and buoyant density of the mercury atom and its affinity for free sulfhydryl groups (on proteins or chromatographic supports) would confer upon modified polymers unique physical properties which can be exploited.

We recently reported the preparation of covalent mercurinucleotides of cytosine, uracil, and 7-deazadenine (Dale et al., 1973). The nucleoside 5-triphosphates of these compounds were, in the presence of appropriate mercaptans, excellent substrates for numerous nucleic acid polymerases. Although covalently mercurated polynucleotides can be prepared enzymatically, the mild reaction conditions used for synthesizing the modified nucleotides suggested that direct polymer mercuration could be achieved as well. In this and the accompanying paper (Dale and Ward, 1975) we (1) detail the synthesis, structural characterization, and properties of the 5-mercuripyrimidine compounds, (2) describe methods for controlled direct mercuration of DNA and RNA polymers, (3) report some of the physical and biological properties of both enzymatically and directly mercurated polynucleotides, and (4) describe a method for the selective and quantitative fractionation of polynucleotide sequences complementary to any mercurated polymer probe by rapid chromatography of mercurated hybrids on columns of sulfhydryl-Sepharose. The studies reported here demonstrate that the bulky and potentially reactive mercury atoms do not significantly alter the structure of the polynucleotide (the mercury atom is located in the major groove of polymer duplexes) nor do they interfere with the ability of the polymers to interact with polymerases, nucleases, and other polynucleotide binding proteins. Mercuripolynucleotides, therefore, appear to be suitable probes for a variety of structural studies.

Materials and Methods

Nonradioactive nucleosides and nucleotides were purchased from Sigma and P.L. Laboratories. Radiolabeled nucleotides (^3H and ^{32}P) and [^{203}Hg]mercuric acetate were obtained from New England Nuclear Corporation. Poly(U), poly(C), poly(A), poly(G), dinucleoside monophosphates, and calf thymus DNA (Type V) were products of Sigma. *Escherichia coli* and yeast bulk tRNAs were obtained from Schwarz/Mann. Purified yeast phenylalanyl-tRNA was kindly provided by Dr. Alex Rich. Double-stranded RNAs (Reo type 3 (dearing) and the replicative

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[1] Although metal coordination complexes contain "coordinate-covalent" bonds, in this and the following paper the term covalent is used to describe only carbon-bound mercury atoms.

form of Qβ bacteriophage) were the gifts of Dr. Aaron Shatkin and Dr. Dan Kolakofsky, respectively. rRNA was prepared from *E. coli* MRE 600 ribosomes (gift of Margaret Schenkman) and the 23S and 16S peaks were separated by velocity sedimentation in sucrose gradients. T_7 was grown on *E. coli* SY106 and purified by CsCl density centrifugation and the DNA isolated by phenol extraction. Purified fd DNA and R17 RNA were the generous gifts of Richard Anderson and Dr. Joan Steitz, respectively. Mercuric salts, chromatographic resins, and other reagents were obtained from regular commercial sources.

Proton magnetic resonance spectra were recorded on a Joel M-100 spectrometer. We thank David Kabakoff for assistance with these measurements. Chemical shifts were measured relative to an external tetramethylsilane standard.

Ultraviolet spectra were recorded on a Cary 15 spectrophotometer. Routine spectral analyses were done using a Beckman 25K recording spectrophotometer.

Thin-layer electrophoresis was performed on a Brinkman-Desaga TLC apparatus using Eastman-Kodak cellulose plates (13255) without fluorescent indicator. All ascending thin-layer chromatography separations were done on the same type cellulose support. Elemental analyses were performed by Baron Consulting Corporation, Orange, Conn.

Sulfhydryl-Sepharose 6B was prepared according to the procedure of Cuatrecasas (Cuatrecasas, 1970). The resin contained 8.1 μmol of sulfhydryl groups/ml, as determined by titration with 5,5′-dithiobis[2-nitrobenzoic acid] (Ellman, 1959). Thiol/CPG-550, a controlled pore glass bead resin containing 30 μmol of sulfhydryl groups/ml, was purchased from Pierce Chemical Company.

Since the rate and extent of polynucleotide mercuration are, unlike that of the mononucleotides, extremely dependent on the ionic strength of the reaction mixture (see text), polymer mercuration conditions will depend on the level of mercuration desired and on the length of time one wishes to expose the polymer to elevated temperatures. The following procedure, however, has been employed as our general method for quantitative mercuration of pyrimidine nucleotides, although other conditions can be used with equally satisfactory results. The compound to be mercurated is dissolved in sodium acetate buffer (pH 6.0, 0.1 *M*) at a concentration of 0.02 *M*. An equal volume of 0.10 *M* mercuric acetate (dissolved in the same pH 6.0 acetate buffer) is added to the nucleotide solution and the mixture heated at 50° for 3 hr. After cooling, the mercurinucleotides are chromatographed on columns of DEAE-cellulose (bicarbonate) using a linear gradient of triethylammonium bicarbonate as the eluent. The mono-, di-, and triphosphates of Hg-C[2] and Hg-U elute at approximately 0.1, 0.22, and 0.35 *M* salt, respectively. The mercurinucleotide fractions are pooled and desalted by rotary evaporation. After washing several times with methanol, the product is dissolved in water, adjusted to pH 7.0 with dilute ammonium bicarbonate, and stored at −20°. To prepare samples for long term storage or for elemental analysis, the nucleotides were chromatographed on columns of DEAE-cellulose (chloride), eluting with a 0–0.4 *M* gradient of lithium chloride. Fractions containing nucleotide were concentrated by rotary evaporation, and the nucleotide was precipitated by the addition of four volumes of acetone. The precipitates were collected by filtration, washed twice with ethanol–ether (1:4) and twice with ether, then dried in vacuo over sodium hydroxide pellets.

Mercurated pyrimidine nucleosides are only limitedly soluble in aqueous solutions (less than 1–2 mg/ml), unlike the parent compounds, and often precipitate during the course of the reaction. To purify, the reaction mixtures are concentrated 3–6-fold by rotary evaporation, the mercurinucleoside is collected by filtration, washed twice with cold 0.1 *M* NaCl, twice with ethanol, and ether, then dried in vacuo.

Unreacted mercuric ions can be removed by passing the reaction mixture through a small column of Chelex 100 resin (Bio-Rad Laboratories) which has been previously washed with 0.1 *M* sodium acetate buffer (pH 6.0) until pH equilibration has been obtained. Chelex 100 has an extremely high affinity for Hg^{2+} ions, binding approximately 0.7 mequiv/ml of resin, but it does not normally adsorb mercurated nucleotides (R-Hg^+). Since Chelex 100 can catalyze a slow demercuration, it is advisable to test the resin before using it in a routine manner and to keep the resin exposure time to a minimum (15–30 min). Chromatography of reaction mixtures on columns of Sephadex G-10 will remove most unreacted Hg^{2+} ions under conditions where no product demercuration occurs. Final purification is then achieved by DEAE-cellulose chromatography.

An alternative procedure for purification, or for measuring product purity, is to chromatograph the reaction mixtures (after removal of free Hg^{2+} ions) on columns of sulfhydryl-Sepharose or sulfhydryl-glass beads. Mercurinucleotides are quantitatively retained on these resins whereas nonmercurated nucleotides are eluted. After washing the resin with 0.1 *M* NaCl to remove any nonmercurated material, the mercurinucleotides are batch eluted with 0.10 *M* sodium cyanide or 0.1 *M* mercaptoethanol. The nucleotide product is precipitated immediately upon elution with four volumes of ethanol or acetone and dried as above. Prolonged exposure to high concentrations of cyanide or mercaptans should be avoided as they induce reductive demercuration when present in large (50–1000-fold) molar excess over the mercurinucleotide.

Radioactive mercurinucleotides are readily prepared by the above procedures using [^{203}Hg]mercuric acetate. Mercury-203 is a relatively inexpensive isotope ($1.20 per mCi in 100-mCi lots) which emits β and γ radiation of 0.212 and 0.28 MeV, respectively, with a half-life of 46.6 days. Currently available specific activities (4 Ci/mmol) yield nucleotides that give 10^7 cpm/μg in either β or γ counters; however, with isotopic enrichment specific activities of over 10^9 cpm/μg are possible. Mercurinucleotides radiolabeled with ^{203}Hg are suitable for autoradiography (Figure 5) and for nucleotide binding studies.

Elemental analysis of the mercurated products revealed that in each case only one mercury atom per base was introduced. Characterization of the compounds (see "Results") has shown the mercury substituent to be on the 5 position of the pyrimidine ring. Typical analytical results are given below for Hg-U and Hg-UMP, respectively. Calcd for $C_9N_2O_5H_{11}HgCl$: C, 22.54; N, 5.84; O, 20.04; H, 2.30; Hg, 41.87; and Cl, 7.41. Found: C, 22.16; H, 2.40, N, 5.56; Hg, 41.30. Calcd for $C_9N_2O_8H_{10}Hg\ PCINa_2\cdot H_2O$: C 17.41; N, 4.51; H, 1.93; Hg 32.34. Found: C, 17.31; N, 4.70; H, 2.15; Hg, 32.48.

[1] Abbreviations used are: Hg-U, Hg-C, Hg-UMP, Hg-CMP, etc., the 5-mercuri derivatives of U, C, UMP, CMP, etc., as the chloride or acetate salts.

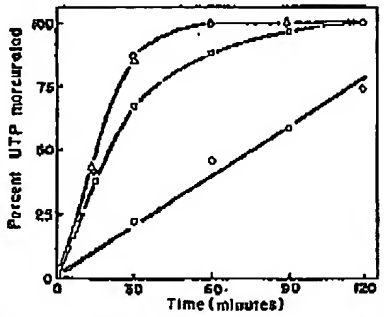

FIGURE 1: Kinetics of UTP mercuration in 0.1 *M* sodium acetate buffer: pH 4.0 (○), pH 5.0 (□), pH 6.0 (○), and pH 7.0 (△). Each reaction (10 ml), run in duplicate, contained 6×10^{-4} *M* UTP and 4×10^{-3} *M* [^{203}Hg]mercuric acetate (specific activity, 1.7×10^{6} cpm/μmol); 1.0-ml aliquots were removed at the indicated times and processed as described under Materials and Methods.

To follow the kinetics of nucleotide mercuration reactions the following protocol was used. Duplicate 10-ml reactions were incubated in the desired buffer containing 5×10^{-4}–5×10^{-3} *N* nucleotide and a fivefold molar excess of [^{203}Hg]mercuric acetate ($1–5 \times 10^{6}$ cpm/μmol). At appropriate times, 1.0-ml aliquots were removed and added to a 1.0-ml suspension of Chelex 100 resin on ice. The resin, prior to use, was adjusted to pH 7.0 by extensive washing with 0.1 *M* sodium acetate buffer (pH 7.0), and tested to be sure it did not catalyze demercuration. The nucleotide–Chelex suspension was stirred or shaken for 5 min, then the resin allowed to settle. The supernatant liquid was removed and treated twice more with Chelex as described above. After the third treatment the resin was removed by filtration, the nucleotide concentration was determined spectrophotometrically, and the ^{203}Hg label not adsorbed to Chelex determined by counting 0.1-ml samples in 3.0 ml of Aquasol using either a Packard scintillation counter or an Intertechnique CG30 automatic γ-counter. Control reactions containing only [^{203}Hg]mercuric acetate were processed in an identical manner to provide the background level of unadsorbed isotope. In all cases greater than 99% of the added [^{203}Hg]mercuric acetate was adsorbed by the Chelex treatment. After subtraction of the background counts the ^{203}Hg cpm/OD_{260} ratio was used to calculate the percentage mercuration. The results of duplicate reactions agreed to within ±2.5%. The covalent nature of the mercuration product was confirmed by electrophoresis at pH 7.5 in the presence and absence of added mercaptoethanol (see text).

Results

Covalent Mercuration of Mononucleotides. Although both purine and pyrimidine nucleotides rapidly form quasi-stable mercury–nucleotide complexes at neutral pH (Yamane and Davidson, 1961; Katz, 1963), only pyrimidine nucleotides undergo facile covalent mercuration. Uracil and cytosine derivatives are quantitatively mercurated within 90 min when heated at 50° in 0.1 *M* sodium acetate buffer (pH's 5.0, 6.0, or 7.0) containing a five- to sixfold molar excess of mercuric acetate. The kinetics of UTP mercuration (Figure 1) are characteristic of all U and C nucleotides. In contrast, less than 2% of thymine, 1% of adenine, and 3% of guanine containing nucleotides are modified after a 24-hr reaction under identical conditions (Table I). Since the purine and thymine nucleotides were not exhaustively purified by chromatographic or electrophoretic means before or after mercuration, the values given represent an upper limit of substitution and may reflect, in part, the purity of the starting material. It is apparent, however, from these studies that nucleotide derivatives of uracil and cytosine react at least 100–200 times faster than other nucleotides. It is interesting to note that pyrimidines with a substituent on the C-5 ring position (thymidine and pseudouridine) are as inactive as purines toward mercuration. The pyrimidine specificity of the mercuration reaction is similar to that seen with thallic chloride catalyzed iodination (Commerford, 1971; Prensky et al., 1973, Scherberg and Refkoff, 1974). However, unlike direct iodination, mercuration proceeds with equal facility on both U and C bases. In addition, whereas extensive uracil hydrate formation occurs in the iodination reaction (Commerford, 1971; Scherberg and Refkoff, 1974) no hydration of the 5–6 double bond occurs during mercuration. Since mercurinucleotides can be rapidly converted to iodonucleotides in high yields (see below), iodination via mercuri intermediates may offer some advantages in the preparation of iodinated nucleotides and polynucleotides.

Table I: Specificity of the Mononucleotide Mercuration Reaction.[a]

Substrate	Percent Mercurated Product: 2-hr Reaction	24-hr Reaction
UTP	100	100
CTP	100	100
ATP	0.45	0.91
GTP	0.58	3.0
TTP	1.7	1.8
Pseudouridine MP		1.3

[a] Reactions (2.0 ml) contained 6×10^{-4} *M* substrate and 4×10^{-3} *M* [^{203}Hg] mercuric acetate (specific activity, 1.7×10^{6} cpm/μmol) in 0.1 *M* sodium acetate buffer (pH 6.0). After heating for the indicated time at 50° the mixtures were cooled on ice, diluted to 10 ml with water, applied to 1 × 3 cm columns of DEAE-cellulose (bicarbonate form), and washed to remove most unreacted mercuric acetate. The nucleotides were batch eluted with 1.0 *M* triethylammonium bicarbonate, desalted by rotary evaporation, and dissolved in 1.0 ml of water. The nucleotide content of each sample was determined spectrophotometrically and a known quantity of the nucleotide electrophoresed at pH 7.5 with and without added mercaptoethanol. The percent mercuration values were calculated from the ^{203}Hg radiolabel associated with uv absorbing material after mercaptan treatment. Similar values were obtained by the Chelex 100 adsorption techniques described under Materials and Methods.

Although purine bases react extremely slowly at neutral pH, they can be mercurated in low yield (10–20%) by refluxing in 50% acetic acid for extended periods (18–24 hr). These extreme reaction conditions, however, preclude direct mercuration of purine compounds with labile pyrophosphate or phosphodiester linkages. Although the normal purine nucleotides are poor substrates for mercuration, the 7-deazapurine analogs of A and G are mercurated as readily as the pyrimidines (Dale et al., 1973).

Mercuric acetate was selected as the mercurating agent because it is (1) highly reactive (Makarova and Nesmeyanov, 1967), (2) highly soluble in aqueous solutions, (3) readily available, and (4) inexpensive. Other mercuric salts are, however, suitable agents. These include mercuric nitrate, mercuric perchlorate, mercuriacetamide, and mercur-

Table II: Effect of Temperature, Buffer Concentration, and pH on the Mercuration of UTP.[a]

Buffer	Buffer Concn (M)	Temp (°C)	Reaction Time (min)	UTP Covalently Mercurated (%)
Sodium acetate, pH 6.0	0.10	37	15	20
pH 6.0	0.10	50	15	38
pH 6.0	0.10	60	15	72
pH 6.0	0.005	50	90	100
pH 6.0	0.05	50	90	98
pH 6.0	0.50	50	90	97
Sodium acetate, pH 4.0	0.1	50	30	26
pH 5.0	0.1	50	30	67
pH 6.0	0.1	50	30	84
pH 7.0	0.1	50	30	86
Tris-acetate, pH 6.0	0.1	50	30	7.5
pH 7.0	0.1	50	30	10.7
pH 8.0	0.1	50	30	9.2

[a] The UTP and [^{203}Hg] mercuric acetate concentrations were the same as those given in the legend to Figure 1. The reactions were terminated and processed as described under Materials and Methods.

itrinitromethane, all of which function with an efficiency similar to that of mercuric acetate. Mercuric oxide and mercuric sulfate are poor reagents since they are only slightly water soluble. Mercuric halide salts (e.g., $HgCl_2$, $HgBr_2$) and mercuric cyanide are essentially inactive; no appreciable level of covalent mercuration could be seen in our test systems even after extensive reaction times.

To minimize the hydrolytic degradation of potential substrates we have chosen reaction conditions which afford high yields when run at or near neutral pH and at relatively low temperatures (50° or less). The rate of mercuration can, however, be increased by utilizing higher temperatures or by increasing the mercuric acetate/nucleotide ratio. As shown in Table II, the mercuration of UTP, which is typical of both U and C monomers, exhibits a temperature coefficient of approximately 1.9 and a pH optimum near pH 7.0.

Mercuration reactions are done in buffered aqueous solutions to prevent the mixtures from becoming acidic as the reaction ($R\text{-}H + HgX_2 \rightarrow R\text{-}HgX + HX$) proceeds. Sodium acetate, sodium citrate, potassium citrate–phosphate, and borate–sodium hydroxide buffers have been found satisfactory. Buffers containing amine salts or halide ions significantly reduce or totally inhibit the reaction. For example, mercurations done in Tris-acetate buffer (pH 6.0) proceed at one-tenth the rate of reactions carried out in an equivalent concentration of sodium acetate (pH 6.0) buffer (Table II). Tris-chloride, glycine-acetate, and glycine-NaOH buffers almost totally inhibit mercuration. Although the buffer concentration can be varied considerably (0.0005–0.50 *M*) without significantly affecting mononucleotide mercuration (Table II), changes in the ionic strength of the reaction profoundly influence both the rate and extent of polynucleotide mercuration (see below). The use of buffers of low ionic strength (<0.02 *M*) decreases the concentrations of both reactants that can be effectively employed, since addition of mercuric salts to concentrated solutions of nucleotide (particularly polynucleotide) in dilute buffer causes an almost immediate precipitation of noncovalent mercurinucleotide salts. Once precipitated the rate of covalent mercuration is significantly reduced. Precipitation problems, occasionally seen with oligo- and polynucleotides containing a high C and/or G content, can be circumvented by increasing the ionic strength or the pH of the reaction buffer.

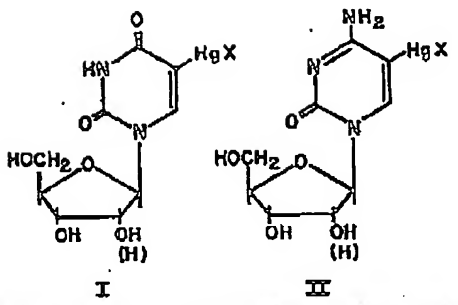

FIGURE 2: Structure of 5-mercuriuridine (deoxyuridine), I, and 5-mercuridcytidine (deoxycytidine), II. The mercury ligand, X, may be Cl^-, CN^-, R-S^-, or other appropriate counterion.

Structural Characterization of Mercurinucleotides. The mono-, di-, and triphosphates of U, C, dU, and dC were converted to the corresponding mercuri derivative as described under Materials and Methods. Although elemental analyses of the mercurinucleotides indicated that each contained a single mercury atom, the site of mercuration had to be established. Since acetoxymercuration reactions proceed via electrophilic substitution (Makarova and Nesmeyanov, 1967), the likely position of attachment is on the C-5 carbon, the most electronegative carbon of the pyrimidine nucleus (Pullman and Pullman, 1969). Three independent methods were used to establish that the products of mercuration are indeed 5-mercurinucleotides (Figure 2).

1. PROTON MAGNETIC RESONANCE (PMR): The mercurinucleotides as originally isolated (see Materials and Methods) contain either bicarbonate or chloride as the counterion to the bound mercury. When solutions of such nucleotide (0.05–0.1 *M* in D_2O) were analyzed by PMR no resonance spectrum could be detected, even at the highest sensitivity settings. In contrast, the corresponding nonmercurated parent compounds gave excellent spectra with the expected chemical shifts. In order to obtain an appreciable PMR signal from the mercurinucleotides, an equivalent molar concentration of a mercaptan, mercaptoethanol, had to be added to the D_2O solutions. Since Hg^{2+} and $R\text{-}Hg^+$ ions can form quasistable complexes with phosphate and aromatic amines, we believe that, in the absence of mercaptan and at high nucleotide concentration, intermolecular interactions generate polymeric charge complexes [PO_4^-–R-Hg^+–PO_4^-–R–Hg^+]n or [R–Hg—N–Ar–Hg—N–Ar]$_n$ which abolishes the monomer resonance. Addition of mercaptoethanol disrupts the polymeric complex by forming the more stable mercurithioethanol nucleotides. The complex forming ability of mercurinucleotides can also be demonstrated spectrally (see below).

Figure 3 shows the downfield PMR spectra of UMP before and after mercuration. Only the chemical shifts assigned (Jardetzky and Jardetzky, 1960; Schweizer et al., 1968) to the H-5 (6.37 ppm) and H-6 (8.40 ppm) of the pyrimidine ring and the H-C_1 (6.41 ppm) of the sugar are illustrated. After mercuration the H-5 doublet has disappeared and the H-6 doublet collapsed to a singlet. Similar changes in the chemical shifts of the H-5 and H-6 protons of cytidine compounds were seen on mercuration. Since the resonances of the other sugar protons were not altered we

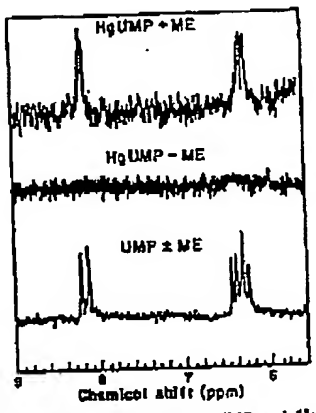

FIGURE 3: Downfield PMR spectra of UMP and Hg-UMP in the presence and absence of 2-mercaptoethanol (ME). Nucleotide concentration was 0.1 *M* in D_2O; mercaptoethanol, where present, 0.1 *M*. See text for discussion.

conclude that the mercury atom is attached to the C-5 ring carbon.

2. MERCURATION OF TRITIATED NUCLEOTIDES. When [^{3}H]CMP and [^{3}H]UMP (tritiated specifically in the C-5 position) are treated with [^{203}Hg]mercuric acetate under the standard reaction conditions, the ^{3}H-radiolabel is quantitatively lost while 1 equiv of ^{203}Hg-radiolabel is acquired. In contrast, no tritium label is removed upon mercuration of [6-^{3}H]uridine. The loss of the C-5 tritium upon mercuration confirms the PMR results and suggests that the reaction proceeds via the classical electrophilic substitution mechanism ($RH + HgX_2 \rightarrow RHgX + HX$).

3. CONVERSION OF HG-UMP AND HG-CMP TO 5-IODO-UMP AND 5-IODO-CMP. Many organomercurials are known to be susceptible to demercuration by a variety of electrophilic reagents, including halogens (Jensen and Rickborn, 1968). Reaction of mercurinucleotides with elemental iodine might therefore be expected to yield the corresponding 5-iodonucleotides. Hg-UMP and Hg-CMP were dissolved in water or 0.05 *M* KI (to enhance the solubility of I_2 in water) and treated with a 50% aqueous-alcoholic solution of I_2. After standing at room temperature for 1 hr the reaction mixtures were extracted three times with chloroform, the residual aqueous solutions filtered through Whatman No. 1 paper, and the filtrates chromatographed on columns of DEAE-cellulose. The reaction products were eluted with triethylammonium bicarbonate (0.12 *M*) and subjected to spectral and chromatographic analyses against authentic samples of 5-iodo-UMP and 5-iodo-CMP. Demercuration of Hg-UMP and Hg-CMP by I_2 gave, in near quantitative yields, nucleotides which were identical in all respects with the corresponding iodinated reference compounds. The demercuration reaction is catalyzed by a variety of electrophiles and we have prepared a number of halogenated and tritiated nucleotide compounds by this general method, the details of which will be published elsewhere.

Properties of Mercurated Nucleotides. Mercurated pyrimidine nucleosides and nucleotides, although normal in many respects, do possess a number of unusual characteristics. For example they form gels when dissolved in water at high concentrations, most likely a consequence of intermolecular interactions of the type described above. Although the viscosity drops on dilution, complex formation still occurs at 10^{-4}–10^{-5} *M* as judged by spectrophotometric measurements. The observed uv absorption of mercurated nucleotides is, unlike that of the parent compounds, salt and temperature dependent. Addition of sodium chloride to a 5×10^{-5} *M* solution of Hg-UMP (in water) increases the overall absorption by 5–15% and induces a very slight blue shift in the long wavelength region of the spectrum. To obtain the maximum increase in absorption a final NaCl concentration of approximately 0.5–1.0 *M* is required. However, strong mercury ligands, such as mercaptoethanol or cyanide (Simpson, 1961), require only a 2–3-fold molar excess. Elevated temperatures (>60°) also give similar small absorption increases which, in contrast to the salt effect, are reversible. The spectral changes resemble those observed on denaturation of polynucleotides. The aggregation which occurs in the absence of an appropriate counterion for the remaining mercuric ionization should, therefore, be taken into consideration when preparing or studying mercurinucleotide solutions.

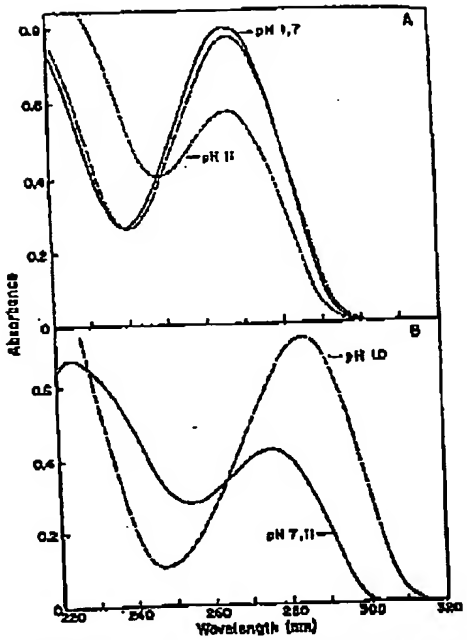

FIGURE 4: The uv spectra of Hg-UMP (A) and Hg-CMP (B) in 1.0 *M* NaCl containing either (a) 0.01 *M* Tris-HCl buffer (pH 7.0), (b) 0.1 *M* HCl, or (c) 0.001 *M* NaOH. (- - -) Spectrum of Hg-UMP in 0.01 *M* Tris-HCl, (pH 7.0) without added sodium chloride. The molar extinction coefficients of Hg-UMP and Hg-CMP at pH 7.0 (in high NaCl) are 10,100 and 9200, respectively.

The spectra of Hg-CMP and Hg-UMP in 1 *M* NaCl (Figure 4) are typical of all mercurated C and U compounds. The absorption maxima occur at longer wavelengths (by 5 nm) than those of the parent pyrimidine nucleotides, although the molar extinction coefficients are essentially identical. The 5-nm spectral difference is independent of the pH at which the spectrum is determined. Although the entire spectrum of Hg-U compounds appears to be red shifted, the Hg-C compounds also lack the broad spectral shoulder in the 230–240-nm region characteristic

Table III: Electrophoretic Properties of Mercurated Nucleotides.[a]

Nucleotide[b]	Electrophoretic Mobility Relative to the Corresponding Nonmercurated Parent Compound			
	pH 3.5		pH 7.5	
	No ME	+ ME	No ME	+ ME
Hg-UMP	0.89	0.65	0.55	0.90
Hg-UDP	0.88	0.84	0.67	0.92
Hg-UTP	0.90	0.87	0.65	0.94
Hg-CMP	0–0.10	0.80	0.40	0.88
Hg-CDP	0.80	0.75	0.72	0.92
Hg-CTP	0.94	0.90	0.82	0.95

[a] Electrophoretograms were run on 20 × 20 cm cellulose thin-layer plates for 3 hr at 300 V in either 0.05 *M* sodium citrate buffer (pH 3.5) or in 0.05 *M* ammonium bicarbonate adjusted to pH 7.5 with CO_2. Aliquots of stock mercurinucleotide solutions were removed and treated at room temperature with a tenfold molar excess of mercaptoethanol for 5 min before spotting. Nucleotide spots were localized by uv adsorption or by autoradiography. [b] The electrophoretic properties of mercurideoxynucleotides are identical with the corresponding ribo-compound.

Table IV: Chromatographic Properties of Mercurated Ribonucleotides in 95% Ethanol–Water (70:30).[a]

Compound	R_f Value	Compound	R_f Value
UMP	0.73	CMP	0.70
Hg-UMP	0.37	Hg-CMP	0.16
UDP	0.70	CDP	0.57
Hg-UDP	0.48	Hg-CDP	0.47
UTP	0.66	CTP	0.53
Hg-UTP	0.54	Hg-CTP	0.39

[a] All chromatograms were run in an ascending fashion on cellulose thin-layer plates.

of C derivatives. Addition of strong mercury ligands to salt solutions of mercurated nucleotides do not induce further spectral changes even though they remove the ionic character of the mercury substituent by forming covalent ligand–mercurinucleotides. Although mercuration reactions can be followed spectrally by monitoring the increase in absorption at 290 nm (for U compounds) or 295 nm (for C compounds), caution should be exercised as the noncovalent mercury–nucleotide complexes possess spectra similar to the covalent derivatives. One can distinguish, however, between the two reactions since the noncovalent complexes are disrupted by the addition of CN^- or Cl^- ions (Yamane and Davidson, 1961; Nandi et al., 1965).

Spectrophotometric titrations of Hg-UMP and Hg-CMP demonstrate that the pK_a values of the ionizable ring protons are not significantly altered as a consequence of mercuration. The observered pK_a values were: Hg-CMP, 4.6; CMP, 4.5; Hg-UMP, 9.7; UMP, 9.6. The hydrogen bonding characteristics of mercurated pyrimidine nucleotides should, therefore, be similar to those of the normal pyrimidines. The facility with which mercurated pyrimidine nucleoside 5′-triphosphates are enzymatically polymerized (Dale et al., 1973), and the thermal denaturation profiles of polymer duplexes containing one Hg atom per base pair (Dale and Ward, 1975), support this contention.

Mercurated nucleotides are readily distinguished from the parent compounds on the bases of their chromatographic and electrophoretic properties (Tables III and IV). When electrophoresed at pH 7.5 as the chloride or carbonate salts, the mercurinucleotides exhibit a significantly slower electrophoretic mobility than that of the corresponding nonmercurated nucleotide. Addition of sodium cyanide or mercaptoethanol to the samples prior to electrophoresis, or inclusion of mercaptoethanol in the electrophoresis buffer, increases the mobility of all mercurinucleotides to approximately 85–95% that of their nonmercurated counterparts. Although the mobility of most mercurinucleotides at pH 3.5 is only slightly altered on the addition of mercaptoethanol, the migration of Hg-CMP increases from 0–10 to 80% that of CMP while Hg-UMP migration, surprisingly, decreases significantly. The increase in electrophoretic mobility at pH 7.5 in the presence of a mercaptan (see Table III and Figure 5) is diagnostic of covalent mercurinucleotides. Electrophoresis also provides a convenient method for quantitating the level of Hg^{2+} (or noncovalent mercury–nucleotide complex) contamination in samples containing ^{203}Hg radiolabel. Prolonged exposure of the mercurinucleotides to a large (50–1000-fold) excess of mercaptoethanol or cyanide should, however, be avoided since these ligands can catalyze reductive demercuration. Mercuricytosine derivatives have been found to be considerably more sensitive to this demercuration process than mercuriuracil compounds. Although somewhat labile in the presence of excess reducing agents, mercurinucleotides are quite stable under the conditions of most biochemical or enzymatic assays and tolerate pH extremes and elevated temperatures with little, if any, degradation (Dale et al., 1973).

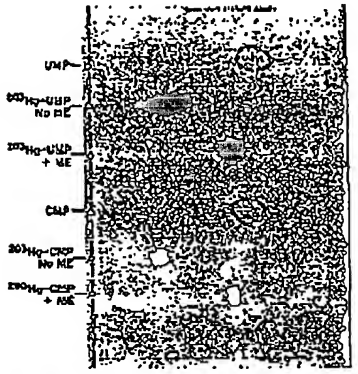

FIGURE 5: Electrophoretic mobility of [^{203}Hg]UMP and Hg-CMP at pH 7.5 in the presence and absence of mercaptoethanol. 0.1 OD_{260} of [^{203}Hg]UMP and [^{203}Hg]CMP (containing 1.1×10^5 and 4.5×10^4 cpm, respectively) were applied to a 20 × 20 cm thin-layer cellulose plate and electrophoresed for 1.5 hr at 300 V. The plate was dried and exposed to Kodak RP/RS4 film for 2 hr before development. UMP and CMP markers were localized by uv adsorption.

Direct Mercuration of Polynucleotides. Since the conditions used for mercurating pyrimidine nucleotides were fairly mild, direct polynucleotide mercuration was examined. Poly(U) was used as the test polymer since it possesses little ordered secondary structure at neutral pH, nor does it form self-duplexes at pH 5.0 or below like poly(C) and poly(A) (Michelson et al., 1967). As shown in Figure 6, poly(U) can be quantitatively mercurated by heating for 2 hr at 50° in 0.005 *M* sodium acetate buffer (pH 6.0) containing a sixfold molar excess of mercuric acetate. The rate of poly(Hg-U) formation exhibits a striking and unexpected dependence on the buffer concentration, the rate being significantly greater in low salt buffers (Figures 6 and 7). This reciprocal relationship is in sharp contrast to the

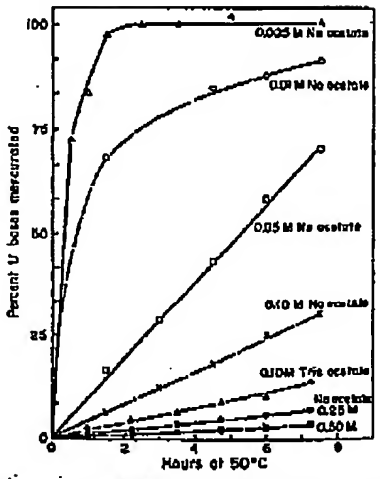

FIGURE 6: The kinetics of poly(U) mercuration at 50° as a function of the buffer concentration. Each 1.0-ml reaction was run in triplicate and, in addition to the indicated concentration of sodium acetate, pH 6.0, or Tris-acetate, pH 6.0, buffer contained 6×10^{-4} *M* poly(U) (dialyzed extensively against water prior to use to remove low molecular weight material) and 4×10^{-3} *M* [^{203}Hg]mercuric acetate (specific activity, 1.1×10^{7} cpm/μmol). Control reactions containing [^{203}Hg]mercuric acetate and polymer were run for each buffer concentration and incubated in parallel at 4°. At the indicated times 0.1-ml aliquots were removed and pipetted into 1.0 ml of ice-cold quench buffer (0.01 *M* Tris-HCl (pH 7.5)–0.1 *M* EDTA–1.0 *M* NaCl). The samples were kept on ice for 10 min, then dialyzed at 4° against TNE buffer (0.01 *M* Tris-HCl buffer (pH 7.5)–0.02 *M* NaCl–0.001 *M* EDTA). A maximum of 40 samples were used per 7 l. of TNE buffer. Dialysis was continued for 48 hr with buffer changes every 12 hr, or until the radioactivity in the control reactions gave only background counts. The polymer content of each sample was determined spectrophotometrically; the ^{203}Hg content obtained by counting duplicate 0.05-ml aliquots, drying on GF/A filters, and using a Packard scintillation counter. The percent of bases mercurated was calculated from the ^{203}Hg cpm/$OD_{260\,m\mu}$ ratio using a molar extinction coefficient of 9800 for poly(U). The results shown are the average of the triplicate reactions; the variability between individual samples was usually less than ±5%.

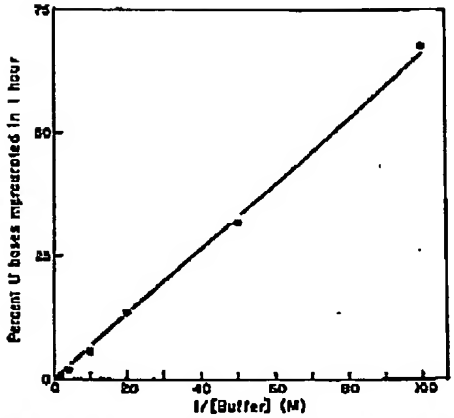

FIGURE 7: The rate of poly(U) mercuration is inversely proportional to the buffer concentrations. Dialyzed poly(U) (6×10^{-4} *M*) and [^{203}Hg]mercuric acetate (1.1×10^{7} cpm/μmol) were reacted for 1 hr at 50° in 0.01, 0.02, 0.05, 0.10, 0.025, and 0.50 *M* sodium acetate buffer (pH 6.0). The reactions were terminated and processed as described in the legend to Figure 6.

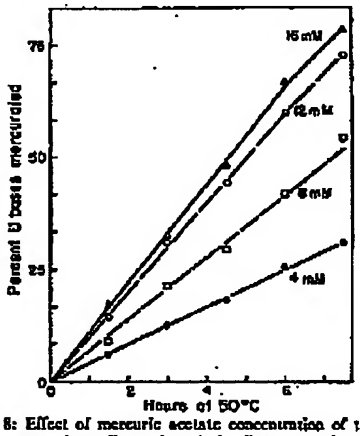

FIGURE 8: Effect of mercuric acetate concentration on the rate of poly(U) mercuration. All reactions (1.0 ml) were run in triplicate in 0.1 *M* sodium acetate buffer (pH 6.0) using 6×10^{-4} *M* dialyzed poly(U) and the indicated concentration of [^{203}Hg]mercuric acetate (9×10^{4} cpm/μmol). Reactions containing the various concentrations of radioactive mercuric acetate but no polymer were run in parallel. Samples were collected and processed as described in the legend to Figure 6.

mercuration of UMP where the rate of reaction is essentially independent of ionic strength (Table II). No convincing arguments can be offered to explain the polynucleotide salt effect. High salt could increase base stacking interactions and thereby alter the electronic character of the uracil base; however, the observation that single- and double-stranded polynucleotides are mercurated at the same rate (see below) seems to make this possibility unlikely. Buffers containing amine salts (e.g., Tris) lower the rate of polymer mercuration but not to the same extent as observed in mononucleotide reactions. Although complete mercuration is obtained within 2 hr under the conditions given in Figure 6, the time of exposure at 50° can be decreased to less than 1 hr by using higher levels of mercuric acetate; the reaction rate increases up to a mercuric acetate/nucleotide ratio of 25–30:1 (Figure 8). Poly(U) mercuration proceeds optimally at neutral pH (pH 6–7) and exhibits a temperature coefficient (Q10) of approximately 2.0–2.2 (Figures 9 and 10). With regard to these parameters, monomer and polymer mercurations are essentially identical. It is apparent from these studies that poly(U) can be extensively modified under conditions where little phosphodiester bond cleavage should occur. Indeed, chromatography of poly(Hg-U) on Sephadex G-200 gave elution profiles which were superimposable on those of the poly(U) starting material. Nucleolytic degradation of poly(Hg-U) and analysis of the resultant nucleotides by chromatography and electrophoresis clearly demonstrate that 5-mercuriuracil bases are the products of mercuration. The characterization and biochemical properties of poly(Hg-U) are presented in the accompanying report (Dale and Ward, 1975).

Polymer mercuration exhibits the same pattern of base specificity as the mononucleotides, as judged by reactions with poly(C), poly(A), poly(G), and poly(T). Since the homopolymers of C, A, and G precipitate in low ionic strength solutions upon the addition of mercuric salts, the reactions were done in 0.1 *M* sodium acetate buffer (pH 6.0) at 50°.

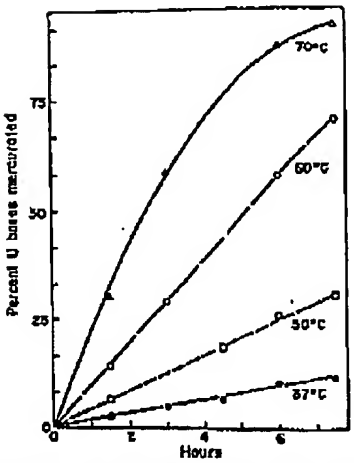

FIGURE 9: Rate of poly(U) mercuration as a function of temperature. All reactions (1.0 ml) were run in triplicate in 0.1 *M* sodium acetate buffer (pH 6.0), with 6×10^{-4} *M* dialyzed poly(U) and 4×10^{-3} *M* [^{203}Hg]mercuric acetate (1.2×10^{7} cpm/μmol). Samples were collected and processed, with appropriate 4° controls, as described in the legend to Figure 6.

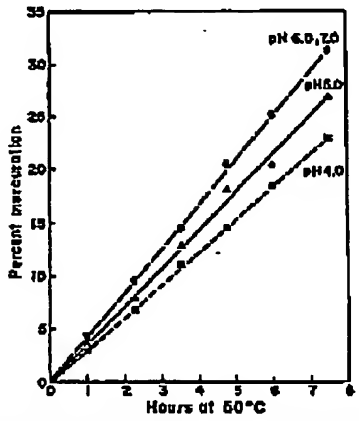

FIGURE 10: Mercuration of poly(U) in 0.1 *M* sodium acetate buffer: pH 4.0 (■), pH 5.0 (▲), pH 6.0 (●), and pH 7.0 (○). Reactant concentrations and work-up are as described in Figure 6.

using only a sixfold molar excess of mercuric acetate. Although these conditions are less than optimal, poly(U) was completely mercurated in 24 hr. In contrast, less than 0.5% of the bases in poly(A), poly(G), and poly(T) were modified (Table V). Poly(C) mercuration proceeded as rapidly as that of poly(U) for several hours. However, the polymer precipitated after 20–40% of the bases had been mercurated. This insolubility problem has prevented our obtaining fully mercurated poly(C) for physical studies. Naturally occurring polynucleotides do not, in general, exhibit the insolubility of the homopolymers and extensive substitution can be achieved in low ionic strength solutions. Figure 11 illustrates the mercuration kinetics of 23S rRNA, bacteriophage R17 RNA, single-stranded fd DNA, and native T_7 DNA. It is apparent from the data that the rate of mercuration with heteropolymers is considerably slower than that observed for poly(U) or poly(C) under comparable conditions. Preliminary studies with dinucleoside monophosphates, UpA, UpG, etc., suggest that there may be a nearest neighbor base effect on the rate of pyrimidine mercuration (R. M. K. Dale and D. C. Ward, unpublished results) which could account, at least in part, for the observed rate difference. It is unlikely that secondary structure plays a dominant role in rate regulation since both native and heat-denatured DNA (T_7 and calf thymus) and RNAs (Reo virus and QβRF) react at identical rates and to the same extent (Table VI). The observation that single- and double-stranded polymers are mercurated with identical kinetics may appear at first somewhat surprising since most direct chemical modifications of polynucleotides, for example, iodination (Commerford, 1971), proceed more rapidly with

Table V: Covalent Mercuration of Homopolymers.[a]

Polymer	Percent Bases Mercurated	
	2 hr	24 hr
Poly(U)	11.3	100
Poly(C)	10.6	ppt.[b]
Poly(A)	<0.05	0.27
Poly(G)	<0.05	0.43
Poly(T)	<0.05	0.13

[a] Reactions (1.0 ml) containing 0.1 *M* sodium acetate (pH 6.0), 4×10^{-4} *M* polymer, and 2×10^{-3} *M* [^{203}Hg]mercuric acetate (1.6×10^{7} cpm/μmol) were incubated at 50°. 1.0 ml of ice cold quench buffer (0.01 *M* Tris-HCl (pH 7.5)–0.1 *M* EDTA–1.0 *M* NaCl) was added to each tube at the indicated times to terminate the reaction. The samples were then chromatographed on 1 × 30 cm columns of Sephadex G-25 using the high salt quench buffer as eluent. Fractions containing polymer were pooled and dialyzed extensively (see legend to Figure 6) before calculating the percent base mercuration from the ^{203}Hg cpm/OD (adsorption maximum) ratio, using the following molar extinction coefficients: poly(U), 9800; poly(C), 7200; poly(A), 10,500; poly(G), 9500; and poly(T), 9200. [b] Poly(C) precipitates from the reaction mixture after 20–40% of the bases are mercurated.

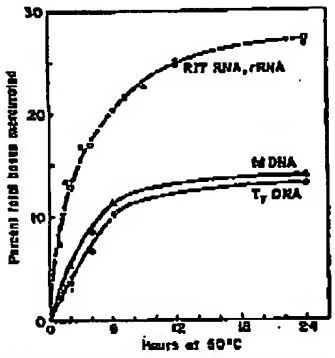

FIGURE 11: Mercuration of R17 RNA (■), 23S rRNA (○), fd DNA (▲), and native T_7 DNA (●). Each 1.0-ml reaction, run in triplicate, contained 0.005 *M* sodium acetate buffer (pH 6.0) and 4×10^{-3} *M* [^{203}Hg]mercuric acetate (1.6×10^{7} cpm/μmol). The polymer nucleotide concentrations were: rRNA and R17 RNA, 4.0×10^{-4} *M*; fd DNA, 4.4×10^{-4} *M*, and T_7 DNA, 6.1×10^{-4} *M*. Concentrations were based on 101 nmol/$OD_{260\,nm}$ for rRNA, R17, and fd DNA and 142 nmol/OD_{260} for native T_7 DNA. Aliquots were removed at the indicated times and processed, with polymer-free controls, as previously described (Figure 6).

Table VI: Single- and Double-Stranded Polynucleotides are Mercurated at the Same Rate.

Polymer	Buffer	Buffer Conen (*M*)	Percent Total Bases Mercurated 1 hr	3 hr	5 hr
Native C.T. DNA	Sodium acetate, pH 6.0	0.005	2.88	7.42	10.0
Native C.T. DNA	Sodium acetate, pH 6.0	0.10	1.40	3.49	5.50
Denatured C.T. DNA	Sodium acetate, pH 6.0	0.10	1.55	3.56	5.46
Native T_7 DNA	Sodium acetate, pH 6.0	0.005	3.52	8.67	12.3
Native T_7 DNA	Sodium acetate, pH 6.0	0.10	1.49	4.30	6.56
Denatured T_7 DNA	Sodium acetate, pH 6.0	0.10	1.67	4.44	6.62
Native QβRF	Sodium acetate, pH 7.0	0.025	7.89	12.7	14.8
Denatured QβRF	Sodium acetate, pH 7.0	0.025	8.63	12.4	16.3
Native QβRF	Tris-acetate, pH 7.0	0.025	2.24	3.60	5.80
Denatured QβRF	Tris-acetate, pH 7.0	0.025	2.30	4.20	6.01
d.s. Reo RNA	Sodium acetate, pH 7.0	0.02	2.85	6.47	8.13
Denatured Reo	Sodium acetate, pH 7.0	0.02	3.20	6.95	8.54
d.s. Reo RNA	Tris-acetate, pH 7.0	0.02	1.14	2.54	3.22

[a] All reactions were incubated at 50° in the presence of an 8–10-fold molar excess of [^{203}Hg] mercuric acetate (1–2 × 10^7 cpm/μmol). The polymer concentrations used were: calf thymus DNA, 1 × 10^{-4} *M*; T_7 DNA, 4.4 × 10^{-4} *M*; Qβ replicative form, 6 × 10^{-4} *M*; Reo RNA, 1 × 10^{-4} *M*. All reactions were processed as described in the legend to Figure 6.

single-stranded polymers. The structure of duplex polynucleotides in the mercuration reactions is not, however, truly native. Noncovalent Hg^{2+}–polymer complexes are formed almost immediately upon addition of mercuric acetate. Although the binding of Hg^{2+} to native DNA (or RNA duplexes) does not induce complete polymer denaturation (Eichhorn and Shin, 1968; Nandi et al., 1965), the bound Hg^{2+} ions must cause a local denaturation or a distortion in the normal helical structure since the Hg^{2+}–base interactions involve amino groups and ring nitrogens (Yamane and Davidson, 1961) which are buried in the native structure. The true substrates in the mercuration of "native" polymers are most likely structurally modified Hg^{2+}–polymer complexes in which the sites for convalent modification are as readily accessible as those of single-stranded polynucleotides.

As shown in Figure 11, under similar reaction conditions ribosomal and R17 RNA react at about twice the rate of fd and T_7 DNA, although all polymers have approximately the same A + T (U) base composition. On the basis of the base specificity shown in Table V one would expect both U and C residues in RNA, but only C residues in DNA, to be modified. Analysis of the chemical and enzymatic degradation products of mercurated polymers confirms this expectation (Dale and Ward, 1975), provided the reaction times are not of extended duration (24 hr or longer). By increasing the mercuric acetate/nucleotide ratio to 30:1 (cf. 10:1 in Figure 11), quantitative modification of all reactible pyrimidine bases can be obtained within 8 hr. However, further incubation at 50° gives a slow but continual rise in the level of polymer bound mercury. Analyses of polymers incubated for 24 hr under such conditions reveal the presence of a new ^{203}Hg-labeled compound which comprises 3–5% of the total bound mercury and possesses electrophoretic properties similar to that expected for 8-mercuri-GMP. The nature of this minor product was not, however, characterized further.

Heteropolymer mercurations, like poly(U) reactions, proceed optimally at pH 6.0–7.0, although the reaction rates do not exhibit as striking a dependence on the buffer salt concentration. For example, a 20-fold decrease in buffer concentration increases the mercuration rate of calf thymus and T_7 DNAs by only twofold (Table VI). An interesting and unexpected salt effect was, however, observed while

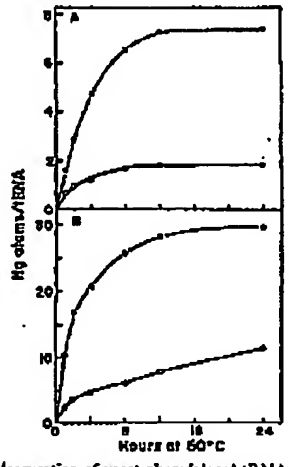

FIGURE 12: Mercuration of yeast phenylalanyl-tRNA (A) in 0.01 *M* (●) and 0.5 *M* (○) Tris-acetate buffer (pH 7.0) and (B) in 0.05 *M* (●) and 0.5 *M* (○) sodium acetate buffer (pH 7.0). The results shown are the average of triplicate determinations. Each reaction (1.0 ml) contained 4.8 OD_{260} (7 nmol) of tRNA (5.2 × 10^{-6} *M* total nucleotide) and [^{203}Hg]mercuric acetate (2.3 × 10^7 cpm/μmol), 2 × 10^{-3} *M* (A); 5 × 10^{-3} *M* (B). Aliquots (0.15 ml) were removed at the indicated times and chromatographed on 1 × 10 cm columns of Sephadex G-25 using quench buffer (see Table V) as the eluent; 0.4-ml fractions were collected and 25 μl counted in Aquasol to locate the tRNA. The peak fractions were pooled and dialyzed against TNE buffer (see Table V). The ^{203}Hg/tRNA ratio was determined as previously described (Figure 6).

studying the mercuration of tRNA. In sodium acetate buffers tRNA (both purified and unfractionated species from *E. coli* and yeast have been tested) undergoes extensive substitution with kinetics similar to those observed for other polymers. In contrast, reactions done in Tris-acetate buffers yield only a limited number of modified bases. Results obtained with yeast phenylalanyl tRNA are illustrated in Figure 12. From the nucleotide sequence data (RajBhandary and Chang, 1968) and the base specificity of the mercuration reaction, it is estimated that yeast $tRNA^{Phe}$ should possess 28 reactible pyrimidines (ribothymidine, pseudouri-

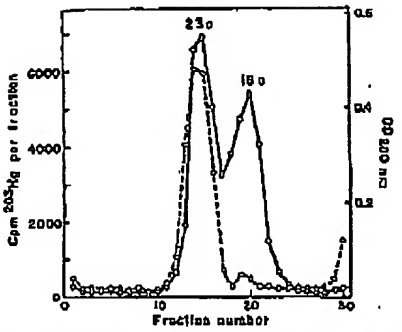

FIGURE 13: Sedimentation profile of 23S rRNA after mercuration of 18% of its pyrimidine bases. Five OD_{260} of 23S RNA was treated with [^{203}Hg]mercuric acetate for 90 min under the conditions given in Figure 11. The reaction was quenched and chromatographed on Sephadex G-25 as described in Table V to remove excess mercuric acetate. 0.1 ml of the RNA peak containing 0.15 OD_{260} (24,000 cpm of ^{203}Hg) was removed, 4.0 OD_{260} (50 µl) of cold (23S + 16S) rRNA added, and the sample centrifuged in a 5–20% sucrose gradient (in 0.01 *M* Tris-HCl buffer (pH 7.5)–0.1 *M* NaCl–10^{-5} *M* mercaptoethanol), for 2.5 hr at 48,000 rpm in an SW 50.1 rotor; 0.15-ml fractions were collected by bottom puncture, 50 µl was counted in 3.0 ml of Aquasol for ^{203}Hg cpm, and the residual sample was diluted to 1.0 ml with water for OD_{260} determinations.

dine, 5-methylcytidine, and dihydrouridine being excluded). Between 28 and 29 Hg atoms/tRNA was introduced when $tRNA^{Phe}$ was reacted for 12 hr in 0.01 *M* sodium acetate buffer (pH 7.0). Increasing the buffer concentration to 0.5 *M* decreased the rate significantly but the reaction does proceed slowly to essentially complete substitution. In contrast, the extent of substitution in 0.05 *M* and 0.5 *M* Tris-acetate buffer (pH 7.0) appears to be limited to about 7.2 and 2.0 Hg atoms/tRNA, respectively. The low level mercuration plateau in 0.5 *M* Tris-acetate buffer has been observed for all tRNA species examined; it occurs in the presence or absence of 10 m*M* Mg^{2+} ions, it is independent of temperature from 30 to 50°, and it is not significantly altered by increasing the Hg^{2+}/nucleotide ratio in the reaction mixture by 20-fold. Schmidt et al., 1973, recently reported that under certain conditions the thallic chloride catalyzed iodination of yeast $tRNA^{fMet}$ would preferentially occur on three cytidine residues; two in the amino acid acceptor stem and one in the anticodon loop. The possibility that a similar type of site specific reaction is occurring when tRNA is mercurated in Tris-acetate buffers is currently under investigation.

Limited mercuration of heteropolymers does not disrupt their structural integrity. The sedimentation profile of 23S rRNA is essentially unchanged after mercuration of 18% of the total pyrimidine bases (Figure 13). Reaction conditions which modify 8% of the C bases in the circular single-stranded fd DNA (1 hr at 50°) cause less than 15% of the molecules to undergo a single phosphodiester bond scission. (We thank Dr. Gerald Bourguignon for analyzing the ratio of circular to linear DNA molecules in the electron microscope). The prolonged heating at 50° (7–8 hr) required for extensive or quantitative mercuration does, however, introduce considerable strand cleavage. Since the introduction of only a small number of mercury atoms is sufficient to give quantitative retention of mercurated polymers on sulfhydryl-Sepharose, structurally intact polymer probes can be prepared for use in the hybridization and selective fractionation procedure to be described (Dale and Ward, 1975).

Discussion

The mercurinucleotides described in this paper represent a new class of nucleotide analogs which have a number of unusual and potentially useful properties. Being organomercurial compounds, they do not possess the same degree of chemical stability as classical nucleotides although they are relatively stable in aqueous solutions free of excess reducing agents. Little, if any, cleavage of the mercury–carbon bond occurs under physiological conditions and only a few percent hydrolysis occurs after standing for 3–4 days at room temperature in 0.01 *M* HCl or 0.01 *M* NaOH. The compounds therefore appear to be sufficiently stable to be utilized as heavy atom derivatives for X-ray crystallographic studies. Indeed, the enzymatic incorporation of a single Hg-CMP residue into the amino acid acceptor stem of tRNA has been achieved (Darling, Dale, and Ward, unpublished results; P. Sigler, personal communication) and crystals of mercurated yeast $tRNA^{fMet}$ obtained (P. Sigler, personal communication). The high affinity of organomercurials for mercaptans (association constants of about 10^{16} compared to 10^3 for acetate, Simpson, 1961) makes the mercurinucleotides convenient "starter" molecules for the in situ synthesis of a variety of nucleotide mercurithioesters. These compounds can be used directly to probe some of the steric parameters of enzyme nucleotide binding sites. By such mercaptan manipulations we have observed that the nucleotide binding sites of template dependent DNA and RNA polymerases are sterically quite different from those of other polynucleotide binding proteins (Dale et al., 1973; Dale and Ward, unpublished results). These observations suggest that mercurinucleotides may have general utility as probes of both protein and polynucleotide structure.

Although sufficiently stable to permit routine biochemical studies, the mercury–carbon bond is extremely sensitive to cleavage by electrophiles and reducing agents. This necessitates certain precautions in their handling. For example, the presence of hydroquinones or other antioxidants in phenol, etc., will rapidly catalyze demercuration. Similarly, when utilizing mercaptan (or cyanide) ligands, the mercaptan/Hg ratio should be maintained as close to unity as experimentally feasible since a large mercaptan excess will also cause demercuration. The lability of the mercury–carbon bond to such agents can, however, be put to useful advantage. Treatment of mercurinucleotides (and mercurated polynucleotides) with I_2, *N*-bromosuccinimide, or [^{3}H]sodium borohydride has been found to rapidly generate the corresponding iodinated, brominated, and tritiated compounds (Dale, Livingston and Ward, manuscript in preparation). The tritiation or radioiodination of polynucleotides via mercuri intermediates is done under very mild conditions and produces no uracil hydrates in RNA. This method should have some utility in the preparation of radiolabeled oligo- and polynucleotides, particularly for enzyme binding or in situ hybridization studies. Some addition properties and applications of mercurated polymers are described in the following paper (Dale and Ward, 1975).

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